Expression of cyclin D1 protein in breast tumors and its relationship with genetic alteration*

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Abstraction **Objectives** To study the expression of cyclin D1 protein in mastosis, early stage breast carcinoma and invasive ductal carcinoma, the value of cyclin D1 detected by immunohistochemistry in the diagnosis of the early breast carcinoma, and the relationship between expression of cyclin D1 protein and genetic alteration. **Metheds** Protein expression of cyclin D1 was detected by immunohistochemistry ,the genetic alteration of cyclin D1 was detected by southern blot. **Results** Cyclin D1 protein was not expressed in breast benign hyperplasia. The expression rate of cyclin D1 was 54% in breast carcinoma;There was no significant difference among early breast carcinoma, primary invasive ductal carcinoma and lymph node metastasis loci; The amplification rate of cyclin D1 gene in breast carcinoma was 16%. **Conclusion** 1.The detection of cyclin D1 protein by immunohistochemistry could be used in the diagnosis of the breast carcinoma.2.The expression of cyclin D1 protein is an early event in the pathogenesis of breast carcinoma and could be maintained stably throughout the progression of breast carcinoma. 3. The expression of cyclin D1 protein was also affected by other factors besides cyclin D1 gene amplification.

Key words Tumor; Cyclins; Genetic alteration; Immunohistochemistry

Hyperplasia is an essential character of malignant tumors. The role of cell-cycle regulators associated with cell proliferation is very attractive in oncogenesis. During the cell cycle, the progression through the G1 phrase into S phrase (DNA synthesis phrase) is essential for the initiation of cell cycle. Cyclin D1, an protooncogene identified at recent years, plays a positive-regulation role in the progression[1]. In this study ,we detected expression of cyclin D1 protein and genetic alteration of cyclin D1 in breast tumors and investigated the relationship between cyclin D1 protein and gene amplification.

MATERIALS AND METHODS

Tissue specimens

122 cases of breast tissues were collected from Qi Lu Hospital of Shandong University. It consist of 40 cases of infiltrating ductal carcinoma (among them 15 cases with lymph node metastasis); 42 cases of early stage carcinoma including 16 cases of intraductal carcinoma and 26 cases of intraductal carcinoma with early infiltration. (the invasive component is less than 10%); 40 cases of mastosis .All samples were fixed in 10% formalin and embedded in paraffin for histological diagnosis and immunohistochemistry. Fresh tissue were obtained in 25 of the 40 cases of infiltrating ductal carcinoma also and stored in liquid nitrogen for DNA analysis .

* Supported by natural science foundation of Shandong province (956208)

Immunohistochemical staining

Immunohistochemical was performed by the labeled streptavidin biotin (LSAB) method using vectastain Elite kit (vector, Burlingame, CA). The monoclonal antibody specific for cylinD1 was used at 1:100 dilution. Sections were placed in plastic jars containing 10 m mol/L citrate buffer and heated in microwave oven for 10 minutes at 675w for antigen retrieval .

Detection of genetic alteration of cyclin D1 by Southern blots

DNA was extracted from 25 cases of fresh carcinoma tissue. Nucleic acids were prepared by the guanidinium thyocyanate method as described [2]. Sample DNA (5ug) were digested with restriction endonuclease and fractionated by electrophoresis on 1% agarose gels .The gels were denatured and DNA transferred to nitrocellulose filter .The filters were prehybridized and hybridized in conditions as described[3]. Filters were dried and exposed at -70℃ with intensifying screen for various peri-

od of times to XAR5 Kodak films .The probe used for detection of cyclin D1 was 18-mer of oligodexoynucleotide complementary to the initial stretch of bases in cyclin D1 mRNA from -1 to +17. The probes was labeled by 32p-dcTP (3000Li mmd-1). β-actin was used as control for DNA loading amount, normal breast tissue near early stage carcinoma was used as normal control.

Statistical analysis

X^2 test was used to evaluated the differences between two groups.

RESULTS

The expression of Cyclin D1 protein in mastosis and breast carcinomas

The cyclin D1 was not expressed in normal breast tissue(Fig 1). The cyclin D1 immunostaining signal was located exclusively in nuclear and variable in terms of staining intensity and the proportion of positive nuclei among the cells of individual cases. 500 tumor cells of each case were counted and defined it the positive case if the proportion of positive cells was more than 10% of the tumor cells. In all 40 cases of mastosis, only weak to undetectable staining was seen and no one was defined positive. In 40 cases of invasive ductal carcinoma, 22 cases were defined positive(Fig 2). The positive rate was 55%. The positive rate of early stage breast carcinoma and lymph node metastatic lesion were 52% (22/42) and 53.3% (8/15) respectively. There was no significant difference among them (table 1). In the 15 cases of patient with lymph node metastasis, 12 cases showed the same cyclin D1 immnostaining pattern in the metastatic lesion with its primary lesion. In the 22 cases of intraductal carcinoma with early stage infiltration, there was a very good correlation between the in situ components and infiltration components. 20 cases showed the same immunostaining pattern, 2 cases showed immunostaining increase .In invasive ductual carcinoma, There was no significant correlation between cyclin D1 protein expression and patient's age, lymph-node matastasis and histological grading (table 2).

Table 1 The expression of cyclin D1 protein in early stage carcinoma(EC), invasive ductal carcinoma (IDC) and lymph node metastasis loci(ML).

cyclin D1	EC	IDC	ML
+	22	22	8
-	20	18	7
	40	40	15

x^2=0.07 P>0.05

Table 2 The relationship of cyclin D1 expression with patience age, status of lymph node(LD) and histological grades(HG) in invasive ductal carcinoma.

cyclin D1	age		LD		HD		
	>60or<35	35-60	positive	negative	Ⅰ	Ⅱ	Ⅲ
+	6	16	7	15	5	10	7
-	5	13	8	10	4	10	4

X^2=0.04 P>0.05 X^2=1.15 P>0.05 X^2=0.47 P>0.05

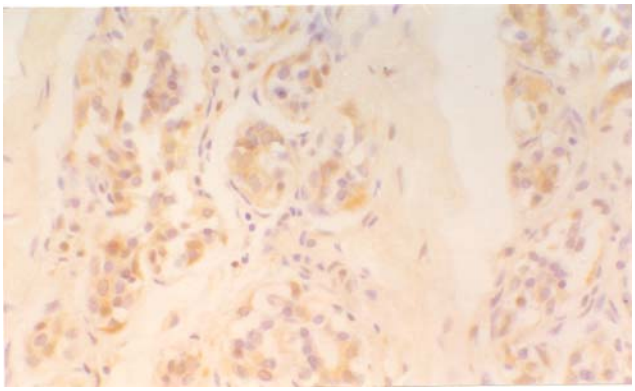

Fig.1 The expression of cyclin D1 protein in normal breast tissue. (LSAB method) ×200.

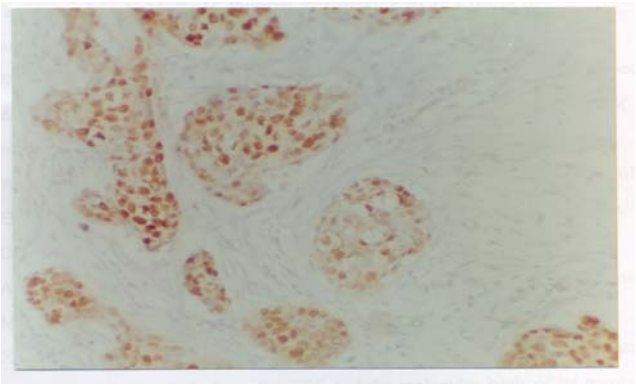

Fig.2 The expression of cyclin D1 protein in invasive ductal carcinoma (LSAB method) . × 200

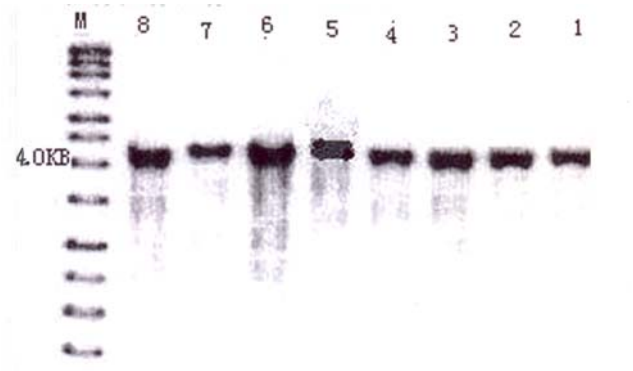

Fig.3 Genetic alteration of cyclin D1 in invasive ductal carcinoma detected by southern blot. M is Marker, 1 is normal control, 6 and 8 are the samples with cyclin D1 gene amplification.

Genetic alteration of cyclin D1 in invasive ductual carcinoma.

Samples from fresh tissue were examined for genetic alteration of cyclin D1 in 25 cases invasive ductual carcinoma. The level of amplification was estimated by densitometric tracing of 4.0 kb cyclin D1 fragment of carcinomas and normal tissue. In EcoR I-digested carcinoma DNAs, 4 cases were observed cyclin D1 gene amplification (2 to 4 fold). No gene rearrangement was observed.(Fig 3). The expression of cyclin D1 protein was positive in the four cases. The ratio of cyclin D1gene amplification(16%) was significantly lower than that of cyclin D1 protein expression (55%).

DISCUSSION

The expression of cyclin D1 in breast carcinoma and breast benign hyperplasia

Cyclin D1 gene is located in the 11q13 region. Amplification of cyclin D1 gene is found in 20%-40% of ovarian and squamous cell carcinomas[4]. Cyclin D1 protein consists of 295 amino acid, it's function in normal cells is to regulate the progression through G1 phrase of the cell cycle by phosphorylation of pRb. When cyclin D1 is combined with cdk4, it will result in the release of transcription factor E2F from pRb. Free E2F mediates transcription of E2f-dependent genes, including DNA polymerase, thymidine kinase[5]. In normal human tissues, the expression of cyclin D1 protein is rather low. In normal breast, most lobules of resting and proliferating breast tissues were observed weak and undetectable staining by immunohistochemistry[6], In our study, no one was positive in 40 cases of mastosis. However, in breast carcinoma, including 40 cases of invasive ductual carcinoma and 42 cases of early stage carcinoma, the positive rate was 54% (44/82).

The immunohistochemical analysis of cyclin D1 protein revealed negative expression in benign breast hyperplasia and positive expression in breast carcinomas. This suggested that the detection of cyclin D1 protein by immunohischemistry is helpful in the diagnosis of early stage breast carcinoma. For the case that can't confirm benign hyperplasia or malignant hyperplasia by microscope, if cyclin D1 protein is positive staining, it has more possibility to be malignant hyperplasia. Some studies showed that overexpression of cyclin D1 was statistically associated with well differentiated tumors[7]. In our study, the expression of cyclin D1 protein has no correlation with patients's age, lymph node metastasis and histological grading.

The expression of cyclin D1 protein throughout breast carcinoma progression.

Bartkora's study [6] revealed a very good correlation between in situ components and invasive components of breast carcinoma. Our results was in according with that. Our study also showed that the positive rate of cyclin D1 protein in early stage carcinoma, invasive ductual carcinoma and lymph node metastases loci was very similar. There was no significant difference among them .A very high degree of accordance was found between carcinoma in situ and its infiltration component (95%). The accordance between primary tumors and lymph node metastases was also high(80%). These results suggested that overexpression of cyclin D1 protein in breast carcinoma may be an early event in breast carcinoma pathogenesis, and it tends to retain stable expression throughout breast carcinoma progression.

The expression of cyclin D1 protein was also controlled by other factors besides gene amplification

In 25 case of EcoRI-digested carcinoma DNAs, 4 cases were observed cyclin D1 gene amplification. No other kind of genetic alteration was observed. These suggested that gene amplification was the main form of genetic alteration in cyclin D1 gene. All these 4 cases showed positive expression of cyclin D1 protein. But the rate of cyclin D1 gene amplification was significantly lower than that of protein expression. This suggested that the expression of cyclin D1 protein was also affected by other factors besides cyclin D1 gene amplification, including alteration in cyclin D1 promoter or abnormal regulation by transcription factors. The transcription factors associated with cell proliferation such as c-myc or c-fos may play a role in it. Besides that, the normal cells in tumor could also affect it, and cause it lower than the case it is.

Cyclin D1 is a proto-oncogene identified at recent years. Our study showed that cyclin D1 was only expressed in breast malignant hyperplasia. The expression of cyclin D1 was an early event in the pathogenesis of breast carcinoma, and the status of expression maintained during the course of infiltration or metastasis. Gene amplification was the main genetic alteration of cyclin D 1 and would result in overexpression of cyclin D1 protein. The study of Sicinski[8] suggested that the proliferation function of steroid including estrogen and progestogen might be mediated by cyclin D1. The study of Worsley[9] showed that the expression of cyclin D1 had correlation with estrogen receptor (ER). The relationship of cyclin D1 with ER and endocrine therapy is

worth to study in the future.

REFERENCES

1. Tamamori-Adachi M, Ito H, Sumrejkanchanakij P, et al. Criti cal role of cyclin D1 nuclear import in cardiomyocyte proliferation. Circ Res, 2003, 92(1):12-19.
2. Wan QH, Qian KX, Fang SG. A simple DNA extraction and rapid specific identification technique for single cells and early embryos of two breeds of Bos taurus. Anim Reprod Sci, 2003, 77(1-2):1-9.
3. Yuan BZ, Zhou X, Zimonjic DB, et al. Amplification and Overexpression of the EMS 1 Oncogene, a Possible Prognostic Marker, in Human Hepatocellular Carcinoma. J Mol Diagn, 2003, 5(1):48-53.
4. Arnold A. The cyclin D1/PRAD1 oncogene in human neoplasia. J Investig Med,1995, 43(6):543-549.
5. Xiao B, Spencer J, Clements A, et al. Crystal structure of the retinoblastoma tumor suppressor protein bound to E2F and the molecular basis of its regulation. Proc Natl Acad Sci U S A, 2003, 100(5):2363-2368.
6. Bartkova J, Lukas J, Muller H, at al. Cyclin D protein expression and function in human cancer. Int J Cancer, 1994, 57: 353~361.
7. Naidu R, Wahab NA, Yadav MM, at al. Expression and amplification of cyclin D1 in primary breast carcinomas: relationship with histopathological types and clinico-pathological parameters. Oncol Rep, 2002, 9(2):409-416.
8. Sicinski P, Joana A, Donaher L, et al. Human D-type cyclin. Cell, 1995; 82:621~630.
9. Worsley S D, Jennings B A, Khail K H, et al. Cyclin D amplification and expression in human breast carcinoma. J Clin Path, 1996, 49:46~50.